A genomic approach to study developmental regulation during oil palm somatic embryogenesis and epigenetic modifications associated with 2,4-D utilization

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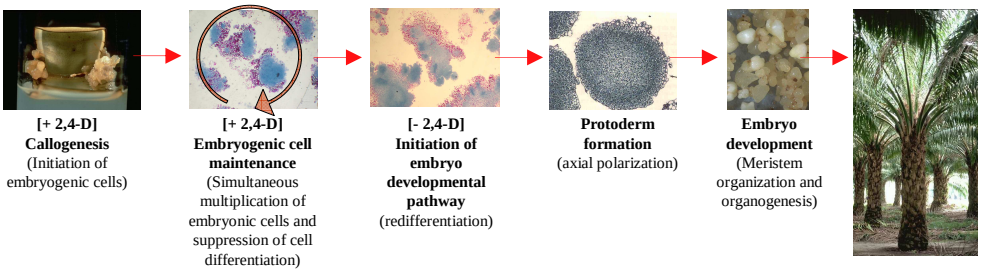
Introduction

Oil palm of the Arecaceae family is a tropical monocotyledonous perennial plant, which is the second most important source of edible vegetable oil worldwide. *In vitro* propagation of elite genotypes of this species is achieved solely by means of somatic embryogenesis (SE) (Aberlenc *et al.* 1999). The inclusion of plant growth regulators in the *in vitro* culture media has been implicated in the generation of somaclonal variation that can give rise to abnormal developmental phenotypes. Such phenomena may often be caused by epigenetic changes to the genome that perturb global DNA methylation and gene expression (Jaligot *et al.* 2000; Tregear *et al.* 2002). We have begun to investigate the possible epigenetic effects of the auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D) on oil palm during SE *in vitro* cell culture. The present study attempts to answer the following questions: (1) Does 2,4-D have an effect on the global methylation of the genome? (2) Are there changes in gene expression that occur during the initiation of the embryogenic pathway upon the removal of 2,4-D?

Indirect Somatic Embryogenesis Culture of Oil Palm (*Elaeis guineensis* Jacq.)



[+ 2,4-D] Callogenesis (Initiation of embryogenic cells)

[+ 2,4-D] Embryogenic cell maintenance (Simultaneous multiplication of embryonic cells and suppression of cell differentiation)

[- 2,4-D] Initiation of embryo developmental pathway (redifferentiation)

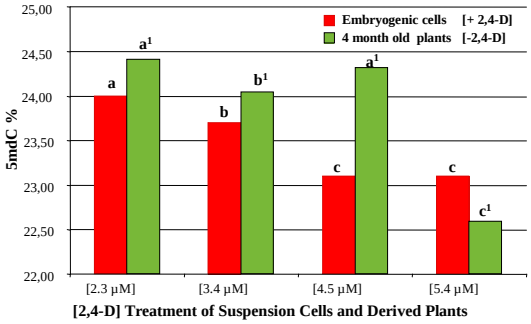
Protoderm formation (axial polarization)

Embryo development (Meristem organization and organogenesis)

Figure 1. The key stages of somatic embryogenesis (SE) of oil palm. The formation of embryogenic cells requires a callogenesis step prior to the initiation of embryo development. Embryogenic cells are maintained in culture for 1 month in a developmentally arrested state in the presence of 2,4-D. The embryo developmental pathway is initiated by the removal of 2,4-D from the culture medium. SE is the only viable method to propagate oil palm *in vitro* but also can be the cause of developmental anomalies that occur at later stages of development (Adam *et al.* 2005).

The Effects of 2,4-D on Global DNA Methylation During *In Vitro* Culture

Figure 2. Analysis of the global methylation status of genomic DNA extracted from embryogenic cell suspensions grown for 30 days with four concentrations of 2,4-D, and young plants derived from these cultures grown on hormone free media. Genomic DNA of embryogenic cells cultivated with 3.4 to 5.4 µM 2,4-D are hypomethylated when compared to those cultivated with 2.3 µM 2,4-D. Genomic DNA of young plants obtained from cultures grown with 5.4 µM 2,4-D remain hypomethylated, while in all other cases, the methylation % returns to normal ratios as previously reported (Jaligot *et al.* 2000).

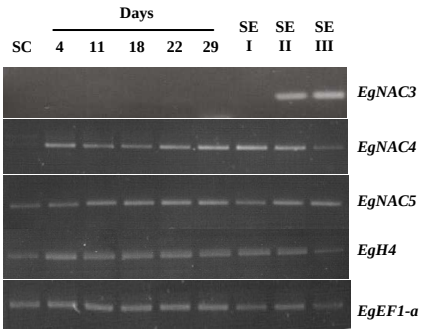


Differential Expression of NAC Genes During *In Vitro* Culture

Figure 3. RT-PCR gene expression analysis of putative NAC domain containing transcription factors during oil palm SE. The *EgNAC3* gene transcript is not detected in suspension cells grown in the presence of 2,4-D (SC) and is expressed only during the later stages of embryogenesis. In contrast, the *EgNAC5* gene transcript is detected in suspension cells grown with or without 2,4-D and throughout SE. Amongst the NAC transcription factors tested, only *EgNAC4* is expressed differentially during the initial phase of SE when the embryogenic pathway is initiated upon removal of 2,4-D.

Legend

SC:	suspension cells grown with 2,4-D	Liquid media
4-29:	4 to 29 days after elimination of 2,4-D	
SEI:	somatic embryos stage I	
SEII:	somatic embryos stage II	Solid media
SEIII:	somatic embryos stage III	



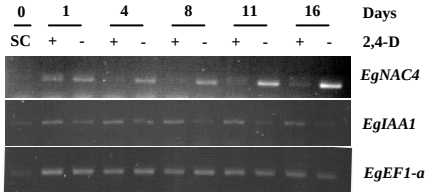
Differential Gene Expression During the Initiation of the Embryonic Pathway

Figure 4. RT-PCR gene expression analysis of candidate genes during oil palm SE. The transcript for the *EgNAC4* gene increases upon initiation of the embryogenic pathway once 2,4-D is removed. In contrast, the transcript for the *EgIAA1* gene, that encodes a protein similar to the auxin induced IAA17 transcriptional regulator, decreases after removal of 2,4-D.

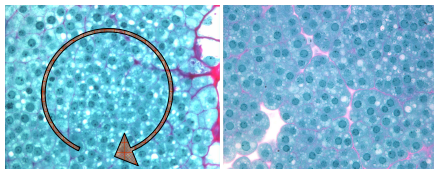
Legend

SC: suspension cells grown with 2,4-D for 30 days

1-16: 1 to 16 days after new media with (+) or without (-) 2,4-D



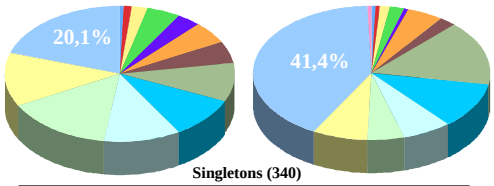
Suppression Subtractive Hybridization Library Construction and Analysis



[+ 2,4-D] Embryogenic cell maintenance S3 Library (189 ESTs)

[- 2,4-D] Initiation of embryogenic pathway S4 Library (183 ESTs)

(372 total ESTs)



Singletons (340)

20,1%	No homology	41,4%
1,1%	Cell wall structure or metabolism	0,6%
2,3%	Cytoskeleton	1,2%
4,6%	Defense and cell rescue	1,9%
3,4%	Secondary and hormone metabolism	0,6%
5,2 %	Membrane transport	4,9%
5,2%	Chromatin and DNA metabolism	2,5%
9,2%	Gene expression and RNA metabolism	15,4%
9,8%	Protein synthesis and processing	11,1%
10,9%	Signal transduction and post-translational regulation	6,8%
14,9%	Primary metabolism	4,9%
12,6%	Unknown function	7,4%
0,6%	Miscellaneous	0,6%

Figure 5. Preliminary analysis of 340 singleton ESTs from the suppression subtractive hybridization (SSH) libraries (each library contains a total of 1,000 clones) constructed from suspension cells grown in the presence (S3 library) or absence (S4 library) of 2,4-D for 15 days. At this time point, changes in cellular characteristics start to become evident (eg. cells are larger, more vaculated and less are undergoing mitotic division) as the embryo developmental pathway is initiated. The ESTs represent genes corresponding to a range of functional categories and will be added to the existing oil palm EST database (Jouannic *et al.* 2005). The most notable difference between the two EST groups is the percentage of genes without similarity to known genes in the available databases.

S3 (+2,4-D) Library		S4 (-2,4-D) Library	
SWI/SNF (remodeling complex)	CD	SNF2 helicase (remodeling complex)	CD
Polycomb (remodeling complex)	CD	minichromosome maintenance	CD
DNA repair AlkB (demethylation)	CD	SET-domain (methylation)	CD
Rad21-2 (regulator of cohesion)	CD	RNA-binding protein	GER
Condensin (mitosis)	CD	NTF2-containing RNA-binding	GER
SET-domain (methylation)	CD	RRM-containing RNA-binding	GER
aux/IAAÖ (auxin response)	GER	DAZ associated RNA binding	GER
zinc-finger TF	GER	glycine-rich RNA binding protein	GER
Putative transcriptional regulator TF	GER	dsRNA-binding	GER
calmodulin-binding TF	GER	RNA-binding protein	GER
NAM (no apical meristem) NAC TF	GER	DNA-directed RNA polymerase	GER
Putative TF	GER	RNA-dependent RNA polymerase	GER
DEAD/DEAH box RNA helicase	GER	transducin / WD-40 repeat (TF complex)	GER
RNA recognition motif	GER	NAM (no apical meristem) NAC TF	GER
glycine-rich RNA binding protein	GER	Egalitarian (cell polarity and fate)	ER
Argonaute (RISC)	GER	Unknown (sea urchin)	ER
DNA-directed RNA pol II	GER		

Figure 6. Summary of the different types of putative regulatory and developmental related cDNAs found in the SSH libraries.

CD:	Chromatin and DNA metabolism
GER:	Gene expression and RNA metabolism
TF:	Transcription Factor
ER:	Embryo Related

Conclusions and Perspectives

As a first step towards determining the molecular basis of oil palm SE and the somaclonal variation events caused by *in vitro* culture conditions, we investigated the methylation status of the genome in relation to different doses of the auxin analogue 2,4-D and the expression of candidate genes during the initiation of embryo development. 2,4-D is commonly used to initiate the formation of cells with embryogenic capacity (Figure 1). Our results indicate that 2,4-D perturbs global DNA methylation of oil palm cells cultured *in vitro* and consequently of plants derived from these cultures (Figure 2). In the case of oil palm, 2,4-D induces the formation of embryogenic cells from explants but also blocks further embryogenic development (Figure 1). Our results suggest that the inhibition of the embryo developmental pathway could also be related to the DNA methylation status influenced by 2,4-D. Here we show that the removal of 2,4-D is also associated with changes in the expression of two putative transcriptional regulator genes, the *EgNAC4* gene transcript accumulates while the *EgIAA1* gene transcript decreases in cells cultured without 2,4-D (Figure 4). The NAC transcription factor family includes members that are involved in shoot meristem formation and development. The *EgNAC4* gene transcript accumulates prior to meristem formation which suggests an early role for this gene in the initiation of embryo development (Figures 1, 3 and 4). In contrast, the *EgIAA1* gene transcript decreases in suspension cells grown without 2,4-D suggesting changes in auxin related transcriptional activity. Auxin is known to play an important role in zygotic embryo axis establishment and is also most likely involved in the initial stages of somatic embryo development. Future work will focus on whether global DNA methylation patterns modulated by 2,4-D are linked to changes in gene expression using of the SSH derived cDNAs in macroarray based gene expression analysis.

References

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